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MUTATION UPDATE

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LAMA2 gene mutation update: Toward a more comprehensive picture of the laminin-α2 variome and its related phenotypes

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Abstract

Congenital muscular dystrophy type 1A (MDC1A) is one of the main subtypes of early-onset muscle disease, caused by disease-associated variants in the laminin-α2 (*LAMA2*) gene. MDC1A usually presents as a severe neonatal hypotonia and failure to thrive. Muscle weakness compromises normal motor development, leading to the inability to sit unsupported or to walk independently. The phenotype associated with *LAMA2* defects has been expanded to include milder and atypical cases, being now collectively known as *LAMA2*-related muscular dystrophies (*LAMA2*-MD). Through an international multicenter collaborative effort, 61 new *LAMA2* disease-associated variants were identified in 86 patients, representing the largest number of patients and new disease-causing variants in a single report. The collaborative variant collection was supported by the LOVD-powered *LAMA2* gene variant database (https://www.LOVD.nl/LAMA2), updated as part of this work. As of December 2017, the database contains 486 unique *LAMA2* variants (309 disease-associated), obtained from direct submissions and literature reports. Database content was systematically reviewed and further insights concerning *LAMA2*-MD are presented. We focus on the impact of missense changes, especially the c.2461A > C (p.Thr821Pro) variant and its association with late-onset *LAMA2*-MD. Finally, we report diagnostically challenging cases, highlighting the

relevance of modern genetic analysis in the characterization of clinically heterogeneous muscle diseases.

KEYWORDS
congenital, *LAMA2*, laminin-α2, locus-specific database, muscular dystrophy, mutation update

1 | BACKGROUND

Laminin-211 is a heterotrimetric cruciform-shaped complex that establishes a stable link between the sarcolemma of muscle fibers and the extracellular matrix, being a major component of the extrasynaptic skeletal muscle basement membrane (BM; Durbeej, 2015). The -211 classification derives from the three specific chains (α2, β1, and γ1), which compose this specific laminin form (Aumailley et al., 2005). Laminin-211 binds to the glycosylated residues of α-dystroglycan (α-DG) and also self-assembles (polymerizes) into networks through its N-terminal domain (Yurchenco, 2015). This supramolecular network connects to collagen IV and to perlecan (heparan sulfate proteoglycan) through nidogens cross-linking (Jones, Dehart, Gonzales, & Goldfinger, 2000). Laminin-211 expression is not confined to skeletal muscle but has also been shown to be expressed in a variety of other tissues, more importantly in peripheral nerve (Schwann cells) and in brain (Yurchenco, 2015). Posttranslational changes have been reported in laminin-211 components. More specifically, laminin-α2 chain was found to undergo cleavage at residue 2580 under specific conditions to generate an N-terminal 300 kDa peptide and a C-terminal 80 kDa peptide, which are subsequently connected through a noncovalent process (Durbeej, 2015).

Disease-associated (pathogenic) variants located in the gene that codes for the α2 chain (*LAMA2*; MIM# 156225) of laminin-211, give rise to a group of diseases collectively designated as *LAMA2*-related muscular dystrophy (*LAMA2*-MD). *LAMA2* maps to chromosome 6q22.33 and spans over 260 kb. It comprises 65 exons and codes for a protein with a molecular mass of approximately 390 kDa (Zhang, Vuolteenaho, & Tryggvason, 1996). The majority of patients with *LAMA2* mutations have a congenital muscular dystrophy (CMD) phenotype classified as type 1A (MDC1A; MIM# 607855). The classical phenotype manifests as neonatal hypotonia or muscle weakness during the first months of life and reduced spontaneous movements (Helbling-Leclerc et al., 1995). As muscle weakness persists during development, it compromises the achievement of normal motor milestones (no cephalic control or inability to sit unsupported) and frequently gives rise to failure to thrive. Other manifestations such as gastroesophageal reflux, aspiration, recurrent chest infections, and even respiratory failure were reported in MDC1A (Jones et al., 2001). Facial muscle weakness, ophthalmoparesis, and macroglossia are also features present in these patients but are often beyond early childhood (Quijano-Roy, Sparks, & Rutkowski, 2012). Other relevant clinical hallmarks of MDC1A include elevated creatine kinase (CK) levels and dystrophic changes (necrosis and regeneration of fibers, chronic inflammation, and fibrosis) recognizable in muscle biopsies of these patients (Tomé et al., 1994). Diagnostically important features are the complete absence of laminin-α2 staining evaluated by immunohistochemistry (IHC) performed in muscle or in skin biopsies (Sewry et al., 1996) using specific antibodies, and typical white matter changes (WMC) in brain detectable by magnetic resonance imaging (MRI; Lamer et al., 1998). WMC are related with alterations in the brain's water content, due to modifications in the maturation and/or function of the blood–brain barrier, and are detectable after the first six months to one year of life (Menezes et al., 2014). Besides WMC, brain structural defects have been reported in patients with laminin deficiency, in an estimated ~4% of *LAMA2*-MD cases (Jones et al., 2001). In some initial studies, performed before *LAMA2* genotyping was available, this association was based solely on laminin staining by IHC (Brett et al., 1998; Martinello, Angelini, & Trevisan, 1998; Philpot et al., 1999; Pini, Merlini, Tomé, Chevallay, & Gobbi, 1996; Sunada, Edgar, Lotz, Rust, & Campbell, 1995; Tsao, Mendell, Rusin, & Luquette, 1998). It is plausible that any dystroglycanopathy could account for the partial laminin deficiency observed in some patients, explaining the diversity of structural brain defects reported. It is nonetheless consensual that primary laminin-α2 deficiency can contribute to structural abnormalities in the cerebral cortex during fetal development. Malformations found in patients with *LAMA2* disease-causing variants includes: (a) cortical dysplasia (Mercuri et al., 1999), (b) changes within the lissencephaly spectrum, namely agyria or pachygyria (Geranmayeh et al., 2010), and (c) polymicrogyria (Vigliano, Dassi, Di Blasi, Mora, & Jarre, 2009).

In a subset of MDC1A cases there is partial laminin-α2 deficiency (reduced/irregular laminin-α2 staining in IHC), which translates into a CMD with a slower disease progression (Geranmayeh et al., 2010; Oliveira et al., 2008). There is some degree of correlation between independent ambulation and IHC status of laminin-α2. The majority of MDC1A patients that do not acquire independent locomotion have complete laminin-α2 deficiency on muscle biopsy, whereas in the majority of cases that are able to walk independently a partial laminin-α2 deficiency has been documented (Geranmayeh et al., 2010).

Further to MDC1A, "milder" *LAMA2*-related phenotypes have been increasingly reported over the past few years. These late-onset *LAMA2*-MD patients are mainly characterized by proximal muscle weakness with onset during childhood, delayed motor milestones, achievement of independent ambulation, and persistently elevated CK levels (Gavassini et al., 2011). Some reports classified these patients as a subtype of limb-girdle muscular dystrophy (LGMD). Patients included in this group may also show muscle hypertrophy, rigid spine syndrome, and pronounced joint contractures which are often more evident in the elbows. In addition to cardiac involvement in a limited number of cases, these clinical features are evocative of Emery–Dreifuss muscular dystrophy (EDMD; Nelson et al., 2015). It should be emphasized that patients with late-onset *LAMA2*-MD still manifest typical brain

WMC, but IHC labeling of laminin-α2 in muscle biopsy may show only very subtle changes.

As laminin-α2 is also expressed in Schwann cells, there is a range of clinical features related with peripheral nerve involvement in *LAMA2*-MD patients. In a particular series of MDC1A patients, the majority had decreased motor nerve conduction, suggesting that peripheral demyelinating neuropathy is a disease feature (Shorer, Philpot, Muntoni, Sewry, & Dubowitz, 1995). Later it was also shown that laminin-α2 related neuropathic abnormalities also included sensory nerves (Quijano-Roy et al., 2004). More importantly, in a milder case of *LAMA2*-MD there was evidence of a myelinogenesis disorder, leading to the assumption that the neuropathy in laminin-α2 deficient cases is actually dysmyelinating (Di Muzio et al., 2003). These changes are more evident in milder *LAMA2*-MD patients (Chan et al., 2014; Deodato et al., 2002; Mora et al., 1996), whereas as in MDC1A presentations the more severe muscle involvement probably masks the subtle neuropathic features of the disease.

In terms of the mutation spectrum of the *LAMA2* gene, four independent studies described cohorts with more than twenty patients (Geranmayeh et al., 2010; Oliveira et al., 2008; Pegoraro et al., 1998; Xiong et al., 2015). The most frequent reported genotypes include variants that create premature termination codons (PTC) in both disease alleles, and are associated with complete deficiency of laminin-α2 in muscle biopsy as well as an MDC1A phenotype. In contrast, missense variants are present in a smaller number of cases and usually correlate with partial laminin-α2 deficiency giving rise to milder phenotypes. The asymmetrical proportion between truncating and non-truncating variants, explains the higher prevalence of MDC1A as compared with other emerging *LAMA2*-related phenotypes.

A relatively high frequency (18.4% of disease-causing variants) of large deletions and duplications in *LAMA2* was also reported. Variants of this sort are detectable by multiplex ligation-dependent probe amplification (MLPA) or array comparative genomic hybridization (array-CGH; Oliveira et al., 2014).

The *LAMA2* locus-specific database (LSDB), which we initiated in 2002, was continuously updated and used to assist the collection of new variants as reported here. Of the 486 unique variants registered to date (December 2017), a total of 61 novel disease-associated variants detected in 86 patients are reported for the first time. Database content is systematically presented and further insights into the genotypes and phenotypes of *LAMA2*-MD are presented.

2 | DEVELOPMENT AND UPDATE OF *LAMA2* LSDB

As part of the work we report the development of a comprehensive database for *LAMA2* variants, an important resource made available for the scientific community since 2002. The LOVD software (Fokkema et al., 2011) was used to store genetic and clinical data, allowing an off-the-shelf LSDB deployment in accordance with international guidelines for the curation and creation of these databases (Celli, Dalgleish, Vihinen, Taschner, & den Dunnen, 2012; Vihinen, den Dunnen, Dalgleish, & Cotton, 2012). The LSDB content was updated and migrated to LOVD version 3.0, being completely redesigned in terms of its database architecture.

Variant data was collected from publications accessed by the curators (64%) or through direct database submissions (36%). Currently (by December 2017), the *LAMA2*-LOVD contains a total of 1,186 of entries (486 unique) identified in a total of 748 individuals. Based on disease impact, these entries comprise: 816 disease-associated variants (309 unique), 317 benign (141 unique), and 53 variants of unknown clinical significance (VUS, 38 unique).

3 | DESCRIPTION OF NOVEL *LAMA2* VARIANTS

A total of 61 novel disease-associated or likely associated variants were identified in the *LAMA2* gene (Table 1), representing more than 20% (61/309) of the total disease variants currently listed in the *LAMA2* LSDB. Variant interpretation followed the standards and guidelines for the classification of sequence variants, proposed by the American College of Medical Genetics and Genomics (ACMG; Richards et al., 2015). The LOVD *LAMA2* database gives two classifications, a Functional classification (column Effect) and a Clinical classification (column ClassClinical). The functional classification indicates the consequences of the variant for the function of the gene/protein (e.g., affects function), the clinical classification the consequences for the individual carrying the variant (e.g., ACMG:5, disease-associated, autosomal recessive [pathogenic]). The summary conclusion of the curators for specific variants, based on all individual observations of the variants, is given in a SUMMARY record. All unpublished variants collected and/or classified in the course of this project can be retrieved from the database using the following link: https://databases.lovd.nl/shared/references/DOI:10.1002/humu.23599.

Variants were identified by different international groups (material and methods in Supporting Information I), which reflects by the diversity of the patients' geographical origins (11 distinct nationalities). Most variants are predicted to be truncating, 20 nonsense type and 23 small frame-shift variants (16 deletions and seven duplications). In addition, this list includes a significant number of variants affecting canonical splice-sites ($n = 13$), the majority located in donor sites (+1 and +2 positions). Due to the inability to obtain proper biological samples or study limitations it was mostly not possible to evaluate their impact at the mRNA level. Thus, the impact of these splice-site variants was evaluated with bioinformatic tools (see Section 3.1), which for all variants indicated unequivocal deleterious effects. One fully characterized was c.819+2T > C, located in the donor splice-site of intron 5. Analysis by RT-PCR followed by sequencing, showed the presence of aberrant transcripts (details in Section 6 and Supporting Information II Figures S1 and S2). In addition to the most prevalent type of variants already stated, the remainder include: (a) two missense variants (one of which might also have an effect on splicing), (b) one in-frame (IF) codon deletion, (c) one deletion-insertion variant, and (d) a large deletion encompassing exons 57 to 65. This large deletion was detected in a homozygous patient with an MDC1A phenotype by array-CGH technique (Supporting Information Figure S3). The 61 new variants were

TABLE 1 Novel pathogenic variants identified in *LAMA2* gene listed in the locus-specific database

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
1	c.47del	Pathogenic	g.129204437delG	r.(?)	p.(Gly16 Alafs*29)	–	1	102376	M	United States	MDC1A	–	Het. / c.2T > C / trans	Pathogenic
1	c.94C > T	Likely pathogenic	g.129204484C > T	r.(?)	p.(Gln32*)	–	1	102361	F	Canada	MDC1A	Deficiency	Het. / c.8245-2A > G / unknown	Likely pathogenic
2	c.164del	Likely pathogenic	g.129371114delA	r.(?)	p.(Asn55 Metfs*16)	–	1	102378	M	Canada	CMD	Deficiency	Het. / ? / unknown	No second pathogenic variant found
2i	c.283+2del	Likely pathogenic	g.129371235delT	r.(spl?)	p.(?)	–	1	102463	F	United States	MDC1A	Deficiency	Het. / c.1609-41_1609-7inv / unknown	VUS [1]
3i	c.396+1G > T	Pathogenic	g.129381042G > T	r.(spl?)	p.(?)	ClinVar (RCV000316746.1); dbSNP (rs770617208); gnomAD (0.0024%)	6	102366	F	United States	MDC1A	–	Het. / c.498G > A / unknown	Pathogenic
								102732	F	Mexico	MDC1A	–	Het. / c.5116C > T / unknown	Pathogenic
								102386	M	United States	MDC1A	–	Hom./ n.a./ n.a.	n.a.
								131976	M	United States	MDC1A	–	Het. / c.6501C > A / unknown	Likely pathogenic
								102478	F	United States	MDC1A	–	Het. / c.6690C > A / unknown	Pathogenic
								36041	M	Lybia	Unknown	–	Het. / c.8586T > G / trans	Pathogenic
4i	c.639+2T > A	Likely pathogenic	g.129419562T > A	r.spl?	p.(?)	–	1	102476	F	United States	MDC1A	–	Het. / c.2049_2050 del / trans	Pathogenic
5i	c.819+2T > C	Pathogenic	g.129465227T > C	r.[640_819 del;640 _1027del]	p.[Ile214_ Arg273del; Ile214 Hisfs*22]	–	3	102735 [2]	M	Portugal	Late-onset *LAMA2*-related MD	Partial deficiency	Het. / c.3976C > T / unknown	Pathogenic

(Continues)

TABLE 1 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
								102736 [2]	F	Portugal	Late-onset *LAMA2*-related MD	–	Het. / c.3976C > T / unknown	Pathogenic
								103207 [3]	F	Portugal	Late-onset *LAMA2*-related MD	–	Het. / c.1854_1861 dup / trans	Pathogenic
7	c.939_940del	Pathogenic	g.129470153_ 129470154del	r.(?)	p.(Cys314 Trpfs*3)	gnomAD (0.0028%)	7	102373	F	United States	MDC1A	–	Het. / c.7732C > T / unknown	Pathogenic
								102382 [2]	F	United States	MDC1A	Partial deficiency	Het. / c.5562+5G > C / unknown	Pathogenic
								132007 [2]	M	United States	MDC1A		Het. / c.5562+5G > C / unknown	Pathogenic
								102396	F	United States	MDC1A	–	Hom. / n.a. / n.a.	n.a.
								132008	F	United States	MDC1A	–	Het./ c.7658delC / unknown	Pathogenic
								132009	M	United States	MDC1A	–	Hom./ n.a./ n.a.	n.a.
								102655	M	United States	MDC1A	Deficiency	Het. / c.7732C > T / unknown	Pathogenic
7	c.991A > T	Likely pathogenic	g.129470205A > T	r.(?)	p.(Arg331*)	gnomAD (0.00041%)	1	102467	M	United States	MDC1A	–	Het. / c.5325dupA / unknown	Pathogenic
12	c.1762del	Pathogenic	g.129513978delG	r.(?)	p.(Ala588 Leufs*11)	Clinvar (RCV000 171527.1); dbSNP (rs78620 5654)	7	102328	F	Saudi Arabia	MDC1A	–	Hom. / n.a. / n.a.	n.a.
								102349	F	Unknown	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
								132010	M	Saudi Arabia	MDC1A	–	Hom. / n.a. / n.a.	n.a.
								132011	M	Saudi Arabia	MDC1A	–	Het. / c.1303C > T/ trans	Pathogenic

(Continues)

TABLE 1 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
								132012 [4]	M	Saudi Arabia	MDC1A	–	Hom./ n.a./ n.a.	n.a.
								132013 [4]	M	Saudi Arabia	MDC1A	–	Hom./ n.a./ n.a.	n.a.
								102363	F	Saudi Arabia	MDC1A	–	Hom. / n.a. / n.a.	n.a.
13	c.1823_ 1824del	Likely pathogenic	g.129571297_ 129571298del	r.(?)	p.(Tyr608*)	dbSNP (rs754600 708); gnomAD (0.00041%)	1	102471	F	United States	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
14	c.2017G > T	Likely pathogenic	g.129573361G > T	r.(?)	p.(Glu673*)	–	1	102460	F	United States	MDC1A	Deficiency	Het. / c.2023_ 2024del / unknown	Likely pathogenic
14	c.2023_ 2024del	Likely pathogenic	g.129573367_ 129573368del	r.(?)	p.(Met675 Aspfs*29)	gnomAD (0.00041%)	1						Het. / c.2017G > T / unknown	Likely pathogenic
17	c.2350dup	Pathogenic	g.129591796dup	r.(?)	p.(Tyr784 Leufs*3)	ClinVar (RCV000 486406.1	1	103206	F	Spain	MDC1A	–	Het. / c.4692_ 4695dup / unknown	Pathogenic
17	c.2383G > T	Likely pathogenic	g.129591829G > T	r.(?)	p.(Glu795*)	dbSNP (rs149896 793); ESP (0.01%); gnomAD (0.00041%)	1	102397	F	United States	MDC1A	–	Het. / c.4761dupT / unknown	Likely pathogenic
17i	c.2450+4A > G	Likely pathogenic	g.129591900A > G	r.(spl?)	p.(?)	–	2	103191	F	Portugal	MDC1A	Partial deficiency	Het. / c.8244+1G > A / unknown	Pathogenic
								103972	M	Portugal	Late-onset *LAMA2*-related MD	–	Het. / c.7750-1713_7899-2154del / unknown	Pathogenic
18i	c.2538-1G > A	Likely pathogenic	g.129608991G > A	r.spl?	p.(?)	–	2	102547	F	United States	MDC1A	Deficiency	Het. / c.3735+2T > A / unknown	Likely pathogenic
								132014	F	United States	MD	–	Het. / ? / unknown	No second pathogenic variant found
21	c.2875C > T	Pathogenic	g.129618848C > T	r.(?)	p.(Gln959*)	–	1	102661	F	Saudi Arabia	MDC1A	–	Hom. / n.a. / n.a.	n.a.

(Continues)

TABLE 1 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
23	c.3338_ 3345dup	Likely pathogenic	g.129634169_ 129634176dup	r.(?)	p.(Thr1116 Glnfs*26)	–	1	102385	M	United States	MDC1A	–	Het. / c.6207C > A / unknown	Likely pathogenic
23	c.3372dup	Likely pathogenic	g.129634203dup	r.(?)	p.(Cys1125 Metfs*4)	–	1	103970	U [6]	Portugal	LGMD/ EDMD [6]	–	Het. / c.2461A > C / unknown	Pathogenic
24	c.3472A > T	Likely pathogenic	g.129635860A > T	r.(?)	p.(Lys1158*)	–	1	102324	F	United States	Unknown	–	Het. / ? / unknown	No second pathogenic variant found
24	c.3520C > T	Pathogenic	g.129635908C > T	r.(?)	p.(Gln1174*)	–	1	103127	F	Spain	MDC1A	–	Het. / c.3976C > T / trans	Pathogenic
25	c.3560_ 3569del	Pathogenic	g.129636625_ 129636634del	r.(?)	p.(Thr1187 Metfs*9)	–	1	102486	F	Canada	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
25i	c.3735 +2T > A	Likely pathogenic	g.129636802T > A	r.spl?	p.(?)	–	1	102547	F	United States	MDC1A	Deficiency	Het. / c.2538-1G > A / unknown	Likely pathogenic
26	c.3829C > T	Likely pathogenic	g.129637000C > T	r.(?)	p.(Arg1277*)	–	1	102383	M	Canada	MDC1A	Deficiency	Het. / c.4654G > A / trans	VUS [1]
27	c.4002T > G	Pathogenic	g.129637260T > G	r.(?)	p.(Tyr1334*)	–	1	102535	F	United States	MDC1A	–	Het. / c.7658delC / unknown	Pathogenic
27	c.4049del	Pathogenic	g.129637307delG	r.(?)	p.(Arg1350 Hisfs*12)	–	2	102663	M	United States	MDC1A	Deficiency	Het. / c.2049_2050 del / trans	Pathogenic
								102401	F	United States	MDC1A	–	Het. / c.5562+5G > C / trans	Pathogenic
29	c.4261C > T	Pathogenic	g.129649507C > T	r.(?)	p.(Gln1421*)	–	1	102662 [4]	M	United States	MDC1A	–	Het. / c.283+1G > A / trans	Pathogenic
30	c.4348C > T	Pathogenic	g.129663524C > T	r.(?)	p.(Arg1450*)	ClinVar (RCV000171401.1); dbSNP (rs200923373); gnomAD (0.0012%)	1	102364	M	United States	MDC1A	Deficiency	Het. / c.2049_2050 del / trans	Pathogenic
33	c.4761dup	Likely pathogenic	g.129687407dupT	r.(?)	p.(Arg1588 Serfs*20)	–	1	102397	F	United States	MDC1A	–	Het. / c.2383G > T / unknown	Likely Pathogenic

(Continues)

TABLE 1 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
34	c.4941del	Likely pathogenic	g.129691117delG	r.(?)	p.(Met1647 Ilefs*5)	–	1	102353	M	United States	Father of affected child (carrier study)	–	Het. / n.a. / n.a.	n.a.
35	c.5050G > T	Pathogenic	g.129704357G > T	r.(?)	p.(Glu1684*)	ClinVar (RCV0000 78775.3; RCV000 177827.2): dbSNP (rs2016 32009)	1	131883	M	Italy	MDC1A	Deficiency	Het. / c.2901C > A / trans	Pathogenic
35i	c.5072-1454_5154 delinsAGA TTGCC	Likely pathogenic	g.129711182_ 129712718 delins AGATTGCC	r.spl?	p.(?)	–	1	102381	M	United States	MDC1A	–	Hom. / n.a. / n.a.	n.a.
36	c.5132del	Pathogenic	g.129712696delA	r.(?)	p.(Glu1711 Glyfs*14)	–	1	102658	M	United States	MDC1A	–	Het. / c.363C > A / trans	Pathogenic
36	c.5134_ 5153del	Pathogenic	g.129712698_ 129712717del	r.(?)	p.(Arg1712 Glufs*4)	–	1	111376	M	Turkey	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
36	c.5182del	Likely pathogenic	g.129712746delC	r.(?)	p.(Leu1728*)	–	1	102358	F	United States	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
37	c.5259del	Pathogenic	g.129714214delA	r.(?)	p.(Val1754*)	–	1	102469	F	Israel	MDC1A	Deficiency	Het. / c.7147C > T / trans	Pathogenic
37	c.5263A > T	Pathogenic	g.129714218A > T	r.(?)	p.(Lys1755*)	–	1	103189	M	Iran	MDC1A	Deficiency	Het. / c.6501C > G/ unknown	Pathogenic
41	c.5914C > T	Pathogenic	g.129748945C > T	r.(?)	p.(Gln1972*)	ClinVar (RCV00 0078782.3, RCV0001 78452.1); dbSNP (rs39 8123378)	4	102365	M	United States	Unknown	–	Hom. / n.a. / n.a.	n.a.
								102384	M	United States	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
								132015 [4]	F	United States	Unknown	–	Het. / ? / unknown	No second pathogenic variant found

(Continues)

TABLE 1 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
								102461	F	United States	MDC1A	–	Hom. / n.a. / n.a.	n.a.
42	c.5998del	Likely pathogenic	g.129759820delA	r.(?)	p.(Thr2000 Profs*3)	–	1	102362	M	United States	MDC1A	Deficiency	Het. / c.7147C > T / unknown	Pathogenic
43	c.6207C > A	Likely pathogenic	g.129762082C > A	r.(?)	p.(Tyr2069*)	dbSNP (rs1433 43647); ESP (0.02%)	1	102385	M	United States	MDC1A	–	Het. / c.3338 _3345dup / unknown	Likely pathogenic
43	c.6266del	Pathogenic	g.129762141delA	r.(?)	p.(Asn2089 Thrfs*14)	–	1	102371	F	Mexico	MDC1A	Deficiency	Het. / c.2962C > T / trans	Pathogenic
45i	c.6429+ 1G > T	Likely pathogenic	g.129766967G > T	r.spl?	p.(?)	gnomAD (0.0032%)	2	102400	M	United States	MDC1A	Deficiency	Het. / c.2901C > A / unknown	Pathogenic
								132016	M	United States	Unknown	Partial deficiency	Hom./ n.a./ n.a.	n.a.
46	c.6501C > G	Pathogenic	g.129774204C > G	r.(?)	p.(Tyr2167*)	–	1	103189	M	Iran	MDC1A	Deficiency	Het. / c.5263A > T/ unknown	Pathogenic
47	c.6588dup	Likely pathogenic	g.129775314dupT	r.(?)	p.(Ile2197 Tyrfs*5)	gnomAD (0.00041%)	1	102379	F	United States	MDC1A	Partial deficiency	Het. / c.7571A > T/ unknown	VUS [1]
49	c.6979G > T	Pathogenic	g.129781456G > T	r.(?)	p.(Gly2327*)	–	1	103192	M	Iran	MDC1A	–	Hom. / n.a. / n.a.	n.a.
51	c.7297C > T	Likely pathogenic	g.129786431C > T	r.(?)	p.(Gln2433*)	–	1	102334	F	United States	MDC1A	Deficiency	Het. / c.35T > G / unknown	Pathogenic
54	c.7491del	Likely pathogenic	g.129799877delA	r.(?)	p.(Asp2498 Ilefs*49)	–	1	102480	M	United States	MDC1A	Deficiency	Het. / c.8244 +3_8244+6 del / unknown	Likely pathogenic
54i	c.7572+ 1G > A	Pathogenic	g.129799959G > A	r.spl?	p.(?)	–	2	111379	F	Germany	Late-onset *LAMA2*-related MD	–	Het. / c.245A > T / unknown	VUS [1]
								102548	F	United States	MDC1A	Deficiency	Het. / c.2T > C / trans	Pathogenic
56i	c.7898+ 2T > G	Likely pathogenic	g.129807769T > G	r.spl?	p.(?)	–	1	111374	M	Turkey	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
56i_ 65_	c.7898+732 _*39282del	Likely pathogenic	g.129808499_ 129876774del	r.(?)	p.(?)	–	1	102475 [5]	M	Saudi Arabia	MDC1A	–	Hom. / n.a. / n.a.	n.a.

(Continues)

TABLE 1 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Number of entries in LSDB	Patient-ID in LSBD	Gender	Geographic origin	Phenotype	IHC for laminin-α2 in muscle/ fibroblasts	Zygosity/ 2nd variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
58i	c.8244+ 1G > C	Likely pathogenic	g.129813629G > C	r.spl?	p.(?)	–	1	102380	M	United States	MDC1A	–	Hom. / n.a. / n.a.	n.a.
58i	c.8244+2dup	Likely pathogenic	g.129813630dup	r.spl?	p.(?)	–	1	111380	F	Saudi Arabia	MDC1A	–	Hom. / n.a. / n.a.	n.a.
59i	c.8357+ 1G > A	Pathogenic	g.129823917G > A	r.spl?	p.(?)	–	1	102391	M	United States	MDC1A	Deficiency	Het. / c.2049 _2050del / trans	Pathogenic
61	c.8556_ 8558del	Likely pathogenic	g.129826353_ 129826355del	r.(?)	p.(Ile2852 del)	ClinVar (RCV0000 78805.4); dbSNP rs39 8123389	1	102737	M	Portugal	MDC1A	Partial deficiency	Het. / c.5234+ 1G > A / unknown	Pathogenic
61	c.8586T > G	Pathogenic	g.129826383T > G	r.(?)	p.(Tyr2862*)	–	1	36041	M	Lybia	Unknown	–	Het. / c.396+1G > A / trans	Likely pathogenic
61	c.8669dup	Pathogenic	g.129826466dupT	r.(?)	p.(Leu2890 Phefs*16)	–	3	102395	M	United States	MDC1A	Deficiency	Het. / c.2370T > A / trans	VUS [1]
								102472	M	United States	MDC1A	Deficiency	Het. / c.2049_ 2050del / trans	Pathogenic
								102369	M	United States	MDC1A	Partial deficiency	Het. / ? / unknown	No second pathogenic variant found
63	c.8947C > T	Pathogenic	g.129833597C > T	r.(?)	p.(Gln2983*)	–	1	111373	M	Turkey	MDC1A	Deficiency	Het. / c.6955C > T / trans	Pathogenic
64	c.9095dup	Likely pathogenic	g.129835624dupA	r.(?)	p.(Ile3033 Aspfs*6)	–	2	102474	M	United States	MDC1A	Partial deficiency	Het. / c.5562+5G > C / unknown	Pathogenic
								132025 [4]	M	United States	Unknown	–	Het./ c.4860G > A / unknown	VUS

Notes. CMD: congenital muscular dystrophy; F: female; Het.: heterozygous; Hom.: homozygous; ID#: identification number; IHC: immunohistochemistry; M: male; MD: muscular dystrophy; MDC1A: congenital muscular dystrophy type 1A; n.a.: not applicable; VUS: variant of unknown significance. [1]: Variant listed in Table 2; [2]: Siblings. Variants were detected by Sanger sequencing, except for: [3]: Whole-exome sequencing (patient ID# 103207), [4]: NGS gene panel (patients' ID#s: 102662, 132012, 132013, 132015, 132025), and [5]: Array-CGH (patient ID# 102475), more details are available in Supporting Information I; [6]: Variant identified through an anonymized screening performed in genetically uncharacterized LGMD/EDMD patients; [a]: According to the ACMG guidelines. References sequences used to describe variants: M_000426.3 and NC_000006.11.

identified in 87 patients (85 families) and in one obligate carrier. In terms of genotypes, a total of 25 patients had homozygous variants. In the remaining 57 cases compound heterozygous variants were found: 52 classified as pathogenic and five VUS. From this cohort, only five cases (5.7%) had incomplete genotyping as only one mutated allele was identified. Four variants were detected in more than three unrelated patients: c.939_940del ($n = 7$), c.1762del ($n = 7$), c.396+1G > T ($n = 6$), and c.5914C > T ($n = 4$). This is explainable by study inclusion criteria and the higher frequency of variants identified in patients of specific populations or ethnic groups still underrepresented in the literature (c.1762del in patients from Saudi Arabia, and the others were found in patients with Hispanic ancestry). Finally, concerning the clinical presentation, the majority of patients were classified as MDC1A, whereas only seven had onset beyond infancy, and achieved independent locomotion. This particular phenotype ("late-onset" *LAMA2*-MD) was seen in patients with splicing variants or missense substitutions, presumably non-truncating alleles, and with partial *LAMA2* deficiency documented in some of the cases.

3.1 | Bioinformatic analysis of novel *LAMA2* variants

The novel *LAMA2* variants, especially those of the missense type and/or predicted to affect splicing, were further assessed resorting to bioinformatic prediction tools. A total of 14 variants fitting into these categories are shown in Table 2. With the exception of homozygotes, all variants are heterozygous and found in combination with a second change known to be disease-associated or likely disease-associated. Since experimental evidence could not be obtained, bioinformatic analysis was pivotal to attempt their classification in terms of pathogenicity.

Listed in Supporting Information III are the *in silico* tools used to evaluate variants, more specifically tolerance predictors and splicing predictors. Considering the extensive list of tools available to evaluate missense variants, we sought to determine which could be most efficient in the case of *LAMA2* variants. The performance measures for binary classifiers, as described by Niroula and Vihinen (2016), were calculated for nine tolerance predictors algorithms. Two control sets of *LAMA2* variants consistently classified in LOVD database as either pathogenic ($n = 29$) or benign ($n = 22$) were used to perform these calculations (Supporting Table). The tools with best performance for our purpose, based on the accuracy and Matthews correlation coefficient, were MutPred2 (Pejaver, Mooney, & Radivojac, 2017), PolyPhen-2 (HumVar; Adzhubei et al., 2010), SIFT (Kumar, Henikoff, & Ng, 2009), and UMD-Predictor (Salgado et al., 2016; Supporting Information Table S1). These algorithms were subsequently applied to evaluate the new missense variants reported in this work (Table 2).

The majority of variants listed in Table 2 were inferred as being of the missense type ($n = 8$). Five variants were consistently classified as deleterious by all four tolerance predictors used, and two variants were classified as deleterious by three out of the four algorithms. The other missense variant was considered deleterious by half of the tolerance predictors.

In four variants, also inferred to be of the missense type, a dual effect was predicted as they could also influence the splicing mechanism. In these, the majority of algorithms tested to evaluate missense variants consistently pointed toward intolerance (all except MutPred2 in three and SIFT in one of the variants) and also indicated an effect on splicing by all tools used, except GeneSplicer in two of the variants.

From the two variants remaining in Table 2, one is an apparently synonymous substitution predicted to create a new acceptor splice-site by three distinct algorithms (all except GeneSplicer) and the other is a large intronic inversion that was predicted to disrupt the canonical acceptor splice-site.

4 | BIOLOGICAL RELEVANCE: CONTENT ANALYSIS OF THE *LAMA2* LSDB

An overview of disease-associated variants found in the *LAMA2* gene is shown in Figures 1 and 2. These may be subdivided as: 59.6% single nucleotide variants (SNV) ($n = 184$ unique; 496 in total), 24.9% small deletions ($n = 77$; 214), 8.7% small insertions ($n = 27$; 62), 6.2% large deletions or duplications ($n = 19$; 42), and two deletion/insertions (0.6%). In terms of their foreseeable impact, the most frequent group is that of variants that cause a PTC. These include nonsense ($n = 79$) and out-of-frame changes (65 deletions, 23 duplications). A total of 79 variants were predicted or experimentally demonstrated to affect splicing. The first and last two conserved nucleotides of introns concentrate the vast majority of splicing variants. It should be highlighted that both PTC-inducing and splice-site variants are widespread throughout the gene with no clear "mutational" hotspots. In terms of distribution throughout the gene, missense variants ($n = 40$, 13% of total disease-associated variants) do not follow a similar pattern; they seem to cluster in specific regions of laminin-α2 (Figure 1). The first group ($n = 11$, 27.5% of missense variants) affect residues located in domain VI corresponding to the N-terminal part of the laminin-α2. A possible explanation is that missense variants located in this region have a detrimental effect on laminin-211 function through the disruption of protein folding and loss of the ability for polymerization into supramolecular networks, that occurs through a cooperative self-assembly process of laminin-211 (Durbeej, 2015; Yurchenco, 2015). A subset of these missense substitutions (namely p.Tyr138His, p.Gln167Pro, p.Leu243Pro, and p.Gly284Arg) are located on the presumed polymerization face near a patch containing the sequence P-L-E-N-G-E, corresponding to residues 208–213 of laminin-α2 (Yurchenco, 2015). These changes were identified in patients with late-onset *LAMA2*-MD with moderately reduced protein levels.

The next cluster consists of missense variants ($n = 10$, 25% of total) that specifically alter cysteine residues, located in one of the three EGF-like repeats (domains V, IIIb, and IIIa), known to establish disulfide bridges. Here, the solenoid-like structure conveyed by these rigid rod-like structures is probably modified in a way that alters the integrity of the connection between the sarcolemma and extracellular matrix mediated by laminin-α2. The last group of missense variants affects residues located in the C-terminal region of the protein that contains a tandem of five laminin G-like (LG) domains—LG1–5. A total of seven disease-associated missense variants (17.5% of all missense) are in LG2, LG3, or LG4 domains. LG4 and LG5 domains mediate

TABLE 2 New *LAMA2* missense changes and other variants possibly affecting splicing based on bioinformatic prediction tools

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Bioinformatic assessment [b]	Patient-ID in LSDB	Gender	Geographic origin	Phenotype	IHC for LAMA2 in mus-cle/skin	Zygosity/ second variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
1	c.112G > A	VUS	g.129204502G > A	r.(?)^ r.(spl?)	p.(Gly38Ser)^ p.(?)	–	Possibly affects function: -Missense variant not tolerated in 3/4 predictors used (all except MutPred2) -Effect on splicing predicted in 4/4 of tools tested	102536	M	Saudi Arabia	MDC1A	Deficiency	Hom. / n.a. / n.a.	n.a.
2	c.245A > T	VUS	g.129371195A > T	r.(?)	p.(Gln82Leu)	–	Possibly affects function: -Missense variant not tolerated in 3/4 predictors used (all except MutPred2)	111379	F	Germany	Late-onset *LAMA2*-related MD	–	Het. / c.7572+1G > / unknown	Pathogenic
3	c.437C > T	VUS	g.129419358C > T	r.(?)	p.(Ser146Phe)	ClinVar (RCV000505761.1); dbSNP (rs143680577)	Possibly affects function: -Missense variant not tolerated in 4/4 predictors used	102470	M	United States	MDC1A	–	Het. / c.6617del / unknown	Pathogenic
5	c.745C > T	VUS	g.129465151C > T	r.(?)	p.(Arg249Cys)	dbSNP (rs376437110); ESP (0.01%); gnomAD (0.0014 %)	Possibly affects function: -Missense variant not tolerated in 4/4 predictors used	102368	M	United States	MDC1A	–	Het. / c.9101_910 unknown	Pathogenic
5	c.818G > A	VUS	g.129465224G > A	r.(?)	p.(Arg273Lys)	ClinVar (RCV000394734.1)	Possibly affects function: -Missense variant not tolerated in 3/4 predictors used (all except MutPred2)	102398	M	United States	MDC1A	–	Het. / c.3976C > T / unknown	Pathogenic
10	c.1326T > G	VUS	g.129498870T > G	r.(?)	p.(Cys442Trp)	–	Possibly affects function: -Missense variant not tolerated in 4/4 predictors used	102360	M	United States	MDC1A	Deficiency	Het. / c.3976C > T / unknown	Pathogenic
								102549	M	United States	MDC1A	–	Het. / c.7658delC / unknown	Pathogenic

(Continues)

TABLE 2 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Bioinformatic assessment [b]	Patient-ID in LSDB	Gender	Geographic origin	Phenotype	IHC for LAMA2 in mus-cle/skin	Zygosity/ second variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
11i	c.1609-41_1609-7inv	VUS	g.129513784_ 129513818inv	r.(spl?)	p.(?)	–	-Effect on splicing predicted in 4/4 of tools tested	102463	F	United States	MDC1A	Deficiency	Het. / c.283+2delT / unknown	Likely Pathogenic
17	c.2370T > A	VUS	g.129591816T > A	r.(?)^ r.(spl?)	p.(790 =) ^p.(?)	–	Possibly affects function: -Effect on splicing predicted in 3/4 of tools tested (all except NNSplice)	102395	M	United States	MDC1A	Deficiency	Het. / c.8669dupT / trans	Pathogenic
23	c.3235T > G	Pathogenic	g.129634066T > G	r.(?)	p.(Cys1079 Gly)	–	Possibly affects function: -Missense variant not tolerated in 4/4 predictors used	102726	F	Portugal	Late-onset *LAMA2*-related MD	Normal	Het. / c.7750-1713_7899-2154del / unknown	Pathogenic
								102718	M	Portugal	Late-onset *LAMA2*-related MD	Partial defi-ciency	Het. / c.3085C > T / trans	Pathogenic
31	c.4523G > A	Pathogenic	g.129670529G > A	r.(?)^r .(spl?)	p.(Arg1508 Lys)^p.(?)	ClinVar (RCV000483171.1); dbSNP (rs770084568); gnomAD (0.00041%)	Possibly affects function: -Missense variant not tolerated in 2/4 predictors (UMD and PolyPhen-2) -Effect on splicing predicted in 4/4 of tools tested	102457 [1]	F	United States	MDC1A	–	Het. / c.2049_2050del / trans	Pathogenic
								102484 [1]	F	United States	MDC1A	–	Het. / c.2049_2050del / trans	Pathogenic
								102656	M	United States	MDC1A	–	Hom. / n.a. / n.a.	n.a.
32	c.4654G > A	VUS	g.129674439G > A	r.(?)	p.(Ala1552 Thr)	dbSNP (rs771891309) gnomAD (0.0012%)	-Missense variant not tolerated in 2/4 predictors (UMD and PolyPhen-2)	102383	M	Canada	MDC1A	–	Het. / c.3829C > T / trans	Likely Pathogenic

(Continues)

TABLE 2 (Continued)

Exon/ Intron	DNA variant (NM_ 000426.3)	Interpretation [a]	DNA variant (NC_ 000006.11) hg19	RNA variant	Predicted effect on protein	External variant databases	Bioinformatic assessment [b]	Patient-ID in LSDB	Gender	Geographic origin	Phenotype	IHC for LAMA2 in muscle/skin	Zygosity/ second variant/ orientation (cis, trans, or unknown)	Interpretation of the second variant
46	c.6548T > G	VUS	g.129774251T > G	r.(?)	p.(Leu2183 Arg)	–	Possibly affects function: -Missense variant not tolerated in 4/4 predictors used	102399	M	Iran	CMD	–	Hom. / n.a. / n.a.	n.a.
47	c.6707G > A	VUS	g.129775433G > A	r.(?)^ r.(spl?)	p.(Arg2236 Lys)^p.(?)	–	Possibly affects function: -Missense variant not tolerated in 4/4 predictors used -Effect on splicing predicted in 3/4 of tools tested (all except GeneSplicer)	103971	U [1]	Portugal	LGMD/ EDMD [2]	–	Het. / c.2461A > C / unknown	Pathogenic
54	c.7571A > T	VUS	g.129799957A > T	r.(?)^ r.(spl?)	p.(Glu2524 Val)^p.(?)	–	Possibly affects function: -Missense variant not tolerated in 3/4 predictors used (all except MutPred2) -Effect on splicing predicted in 3/4 of tools tested (all except GeneSplicer)	102379	F	United States	MDC1A	Partial defi-ciency	Het. / c.6588dupT / unknown	Likely pathogenic

Notes. CMD: congenital muscular dystrophy; F: female; Het.: heterozygous; Hom.: homozygous; ID: identification; IHC: immunohistochemistry; M: male; MD: muscular dystrophy; MDC1A: congenital muscular dystrophy type 1A; n.a.: not applicable; U: unknown; VUS: variant of unknown (clinical) significance; [1]: Siblings; [2]: Variants identified through an anonymized screening performed in genetically uncharacterized LGMD/ EDMD patients; [a]: According to the ACMG guidelines. [b]: More detailed information available in Supporting Information Table S2. References sequences used to describe variants: NM_000426.3 and NC_000006.11.

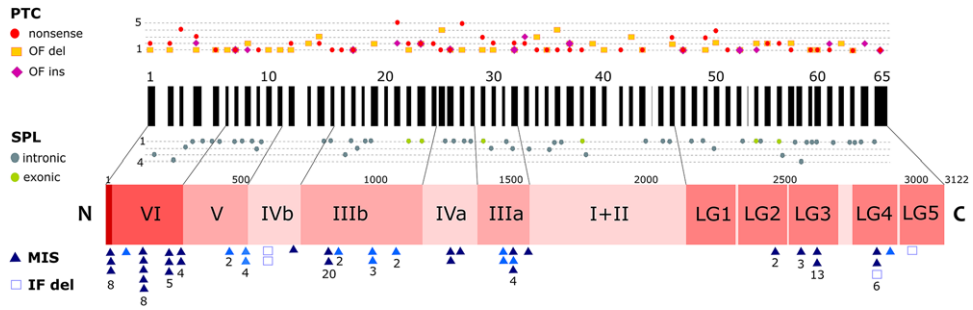

FIGURE 1 Point variants recorded in the laminin-α2 (*LAMA2*)-LOVD. Top layer unveils the number of unique variants that originate premature termination codons (PTC): nonsense, out-of-frame (OF) deletions (DEL) or insertions (INS), per *LAMA2* exon (black rectangles). Middle layer shows splice-site variants (SPL), also indicating the number of unique variants per region: intronic (grey) or exonic (light green). Laminin-211 protein domains: I to VI, and Laminin G-like (LG) are shown in light pink to red boxes, from the C-terminal (C) to N-terminal region (N). Bottom layer displays missense (MIS) changes or single codon in-frame (IF) deletions. Light blue triangles indicate substitution of a cysteine. Variants are clustered per exonic region, and numbers below each symbol indicate the total number of changes

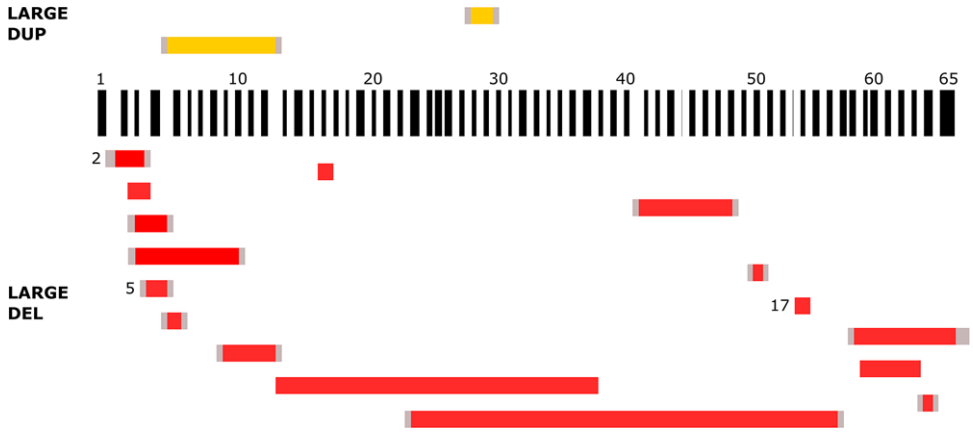

FIGURE 2 Large deletions and duplications listed in the laminin-α2 (*LAMA2*)-LOVD. Large duplications (DUP) are shown in the top layer as yellow rectangles encompassing affected gene regions, and deletions (DEL) are shown in the bottom part of the picture as red rectangles. Black rectangles represent the *LAMA2* gene exons. Grey boxes indicate undetermined breakpoints and numbers the total of entries in the database (otherwise only one entry is present)

the binding of laminin-α2 to the O-linked carbohydrate chains of α-DG, whereas LG2 and LG3 bind to integrin $\alpha 7/\beta 1$. Rare missense variants and IF deletions pose a problem for genetic data interpretation. Four IF codon deletions have been reported so far and, since there are no functional analysis strategies currently available for laminin-α2 variants, their impact remains unclear. A second aspect to be considered, as highlighted before, is that some changes predicted to be missense may instead have an effect on mRNA splicing. In addition to the application of bioinformatics tools used to assess the pathogenicity of missense changes (such as those mentioned in 3.1), it is advisable to consider if their location coincides with the potential hotspots outlined here.

Since our previous assessment (Oliveira et al., 2014) only two novel large deletions have been reported (Bhowmik, Dalal, Matta, Sundaram, & Aggarwal, 2016; Ding et al., 2016), totaling 17 deletions and two duplications (Figure 2). There are two apparent mutational "hotspots" for large deletions, the first region includes exons 3 and 4, and the second is in the 3' end of *LAMA2* gene (exons 56 to 65).

Considering the distribution of disease-associated variants, exons 14, 21, 22, 26, 27, 36, 38, and 56 contain over 25 variant entries. In contrast, seven exons (namely 20, 28, 44, 45, 48, 53, and 58) have no disease-causing variants reported so far. Fourteen disease-associated variants are among the most prevalent in the *LAMA2*-LOVD database, with at least 10 independent entries each (Table 3). The most frequent across different ethnical backgrounds are: c.2049_2050del (p.Arg683Serfs*21), c.3085C > T (p.Arg1029*), and c.3976C > T (p.Arg1326*). Interestingly, these variants are also represented in population variant databases such as gnomAD (ExAC), found in heterozygosity with frequencies ranging from 0.012 to 0.001%. Other variants such as c.1854_1861dup (p.Leu621Hisfs*7), seem to be population- or ethnic group-specific, exhibiting a relatively high frequency (0.23%) within control alleles from the "Latino" population (gnomAD).

5 | CLINICAL RELEVANCE: THE EXPANDING DISEASE SPECTRUM OF *LAMA2*-RELATED MD

5.1 | Genotype–phenotype correlations

The severest end of the spectrum of *LAMA2*-related MD—MDC1A—corresponds to a neonatal onset disease that gives rise to hypotonia and compromised normal motor development. In *LAMA2*-related MD

TABLE 3 List of the most frequent pathogenic variants in the *LAMA2* LSDB (variants with 10 or more entries in LOVD)

Exon/ Intron	DNA variant (NM_000426.3)	DNA variant (hg19)	RNA variant	Predicted effect on protein	Number of independent entries in LOVD	Geographic origin of patients (LOVD)	gnomAD/ExAC data: population, nr alleles/ total alleles (frequency)
13	c.1854_1861dup	g.129571328_129571335dup	r.1854_1861dup	p.Leu621Hisfs*7	12	France, Portugal, Brazil, Spain	Latino: 2/838 (0.23%)
14	c.2049_2050del	g.129573393_129573394del	r.2049_2050del	p.Arg683Serfs*21	42	Several countries	All populations except Ashkenazi Jewish: 34/277,008 (0.012%)
18	c.2461A > C	g.129601216A > C	r.2461a > c	p.Thr821Pro	18	Portugal	–
22	c.3085C > T	g.129621928C > T	r.(?)	p.(Arg1029*)	24	Portugal, Spain, United States	Latino: 1/34,418 (0.003%); European (Non-Finnish): 1/126,676 (0.001%)
26i	c.3924+2T > C	g.129637097T > C	r.3736_3924del	p.Leu1246_Glu1308del	33	Saudi Arabia, Sudan, United States	–
27	c.3976C > T	g.129637234C > T	r.(?)	p.(Arg1326*)	24	Portugal, Sweden, United States, Spain, Denmark	European (Non-Finnish): 14/126,524 (0.001%); European (Finnish): 1/25,788 (0.004%)
32	c.4645C > T	g.129674430C > T	r.[4645c > u, 4580_4717del]	p.[Arg1549*, Cys1527_Val1572del]	10	Australia, Italy, United States	South Asian: 2/30,780 (0.006%)
36i	c.5234+1G > A	g.129712799G > A	r.5072_5234del	p.Val1765Serfs*21	10	Portugal, Canada, United States	Latino: 1/34,376 (0.003%); European (Non-Finnish): 2/126,266 (0.002%)
38	c.5476C > T	g.129722399C > T	r.(?)	p.(Arg1826*)	11	China, Saudi Arabia, United Kingdom	East Asian: 2/17,240 (0.012%); European (Non-Finnish): 5/111,398 (0.0045%)
38i	c.5562+5G > C	g.129722490G > C	r.[5446_5562del, 5562_5563ins5562	p.[Lys1816_Asp1854del, Tyr1855Valfs*24]	14	United Kingdom, United States	European (Non-Finnish): 7/125,864 (0.0056%); European (Finnish): 1/25,408 (0.0039%)
46	c.6488del	g.129774191delA	r.(?)	p.(Lys2163Argfs*12)	15	Qatar, Saudi Arabia, United States	–
55	c.7732C > T	g.129802567C > T	r.(?)	p.(Arg2578*)	12	China, Denmark, Mexico, Russian Federation, United States	Latino: 3/34,380 (0.0087%); European (Non-Finnish): 10/126,598 (0.0079%); East Asian: 1/18,834 (0.0053%); South Asian: 1/30,782 (0.0032%)
55i_56i	c.7750-1713_7899-2154del	g.129805906_129810892del	r.7750_7898del	p.Ala2584Hisfs*8	17	Portugal	–
58i	c.8244+1G > A	g.129813629G > A	r.8076_8244del	p.Pro2693Valfs*12	12	Germany, Portugal, Tunisia, United States	European (Non-Finnish): 1/111,114 (0.0009%)

Note. nr: number.

the locomotion attainment has been considered an important clinical measure of disease severity. In a series of 26 MDC1A patients only two had acquired independent locomotion (Oliveira et al., 2008). Interestingly, all patients that harbored variants inducing PTC in both disease alleles were unable to achieve independent walking. In contrast, the two patients that were able to walk had a missense or a single codon deletion in one of the disease genes. LSDB content and other studies reported in the literature (Geranmayeh et al., 2010) further corroborated our findings. However, there are exceptions to this rule; for example, a patient with a homozygous nonsense variant

(p.Arg1549*) was able to reach ambulation and even climb stairs (Geranmayeh et al., 2010). This particular variant was reported in association with partial deficiency of laminin-α2 in several unrelated patients (Di Blasi et al., 2000; Geranmayeh et al., 2010; Pegoraro et al., 1998). Here, an explanation for this discrepancy is the fact that the variant is located within exon 32 that undergoes alternative splicing (Pegoraro et al., 2000). The exon removal leads to an IF deletion at the mRNA level, thereby restoring the reading frame from the PTC created by the nonsense variant.

Geranmayeh et al. (2010) provided further genotype–phenotype correlations with prognostic clinical implications. Statistically significant differences were identified between patients with complete deficiency and those with partial deficiency of laminin-α2. Patients with absence of laminin-α2 had earlier onset ($P = 0.0073$), lack of independent ambulation ($P = 0.0215$), and were more prone to requiring artificial feeding ($P = 0.0099$) or respiratory support ($P = 0.0354$; Geranmayeh et al., 2010). Within MDC1A, there is a subset of patients with early onset phenotype but a "milder" disease progression and with partial laminin-α2 deficiency. This partial deficiency is often associated with missense variants, IF deletions, and splicing variants (leaky or inducing IF exon-skipping; Allamand & Guicheney, 2002; Quijano-Roy et al., 2012). One of the earliest such cases reported had a homozygous variant (p.Cys996Arg) that affects domain IIb of laminin-α2 (Nissinen et al., 1996).

Despite the general consistency between phenotype, the type of variant and the IHC status, some exceptions have been documented in the literature. These include patients with complete laminin-α2 deficiency and missense variants that achieved independent locomotion (Geranmayeh et al., 2010), although this could be attributed to IHC sensitivity issues. Intrafamilial clinical variability has also been reported, such as that found among patients from one large Kenyan kindred of Asian ancestry. Here, patients shared the same genotype (homozygous missense variant located in the G-domain of laminin-α2) but locomotion was not achieved in all cases (Geranmayeh et al., 2010).

As previously mentioned, a very small fraction of *LAMA2*-MD patients have brain structural defects, which are frequently associated with intellectual disability (ID) and/or refractory seizures (Geranmayeh et al., 2010, Vigliano et al., 2009). However, there are also reports of patients, with these structural defects who, apparently have no seizures or ID. The opposite also holds true in the case of seizures (and to a lesser extent ID) since they have been reported in patients without cerebral structural changes. Based on the reassessment of data available in the LOVD and reported in the literature, no association was found between epilepsy, cognitive function or brain anomalies, and a particular set of *LAMA2* genotypes/variants. The variants found in these cases are diverse in terms of their impact, ranging from those causing PTC to missense changes, and are apparently dispersed with no obvious hotspot along the gene. Furthermore, phenotypical discrepancies have been found in patients sharing with same genotype. For example, two siblings reported by Di Blasi et al. (2001) and case #2 from Nelson et al. (2015) share the same genotype (a homozygous nonsense variant p.Arg744*), but cortical polymicrogyria and lissencephaly were only reported in the latter patient. It is conceivable that other genetic factors besides *LAMA2* variants are contributing to these phenotypes.

Over the last few years there has been a significant increase in reports of late-onset *LAMA2*-related MD patients (Ding et al., 2016; Gavassini et al., 2011; Harris et al., 2017; Kevelam, van Engelen, van Berkel, Küsters, & van der Knaap, 2014; Kim et al., 2017; Løkken, Born, Duno, & Vissing, 2015; Marques et al., 2014; Nelson et al., 2015; Rajakulendran, Parton, Holton, & Hanna, 2011). Most of these patients have heterozygous or homozygous missense or splice variants. Their clinical presentation is also variable but often overlapping with a childhood-onset LGMD, consisting of proximal muscle weakness and delayed motor milestones, but in all cases achieving independent ambulation. Rigid spine syndrome with joint contractures has been also reported in some patients (Nelson et al., 2015).

5.2 | Additional cases of late-onset *LAMA2*-related MD sharing the p.Thr821Pro variant

Phenotypic variability in *LAMA2*-related MD has been clearly underestimated so far, with only a limited number of patients with this later-onset phenotype reported in the literature. As for establishing further genotype–phenotype correlations, the cases are still relatively scarce and there is a vast diversity of genetic defects and/or genotypes, which makes it difficult to stratify patients into homogeneous groups.

To address some of these limitations, and resorting to our large *LAMA*-related MD patient cohort, the clinical and genetic characterization of six additional patients with a late-onset phenotype from four unrelated families is reported (Table 4). They all share the same missense variant: p.Thr821Pro. In five cases the genotype was similar in that, besides this missense substitution, the second allele was a truncating variant: c.7750-1713_7899-2154del (p.Ala2584Hisfs*8) in patients P1 and P2, c.3976C > T (p.Arg1326*) in P3 and P4, and c.1854_1861dup (p.Leu621Hisfs*7) in P5. The sixth patient (P6) represents the first documented case with a homozygous p.Thr821Pro missense variant. Most of these patients were only diagnosed during adulthood, which reflects the diagnostic difficulties concerning non-MDC1A cases. All have a very mild muscle weakness (as compared with typical MDC1A) with lower limb weakness resulting in gait disturbances. In the oldest patient (P6) this weakness culminated in loss of ambulation during the sixth decade of life. In four patients brain MRI was performed (P1, P2, P3, and P6), revealing WMC like those usually found in *LAMA2*-related MD (Figure 3a–c). These findings were pivotal for conducting *LAMA2* gene analysis in three of the cases. Patient P6, who developed dementia over the last 2 years, also had hypothalamus and pons alterations (data not shown). Five patients were subjected to a muscle biopsy. These showed myopathic or dystrophic features (Figure 3d–f), and IHC analysis for laminin-α2 revealed apparently normal labeling ($n = 3$, Figure 3g–i) or partial deficiency ($n = 1$, data not shown).

5.3 | Prevalence of p.Trp821Pro variant in a genetically uncharacterized MD patient cohort

The missense variant p.Trp821Pro is one of the most frequent genetic causes of late-onset *LAMA2*-MD in a population-specific (Portuguese)

TABLE 4 Additional *LAMA2*-related muscular dystrophy patients sharing the p.Thr821Pro variant

Family patient #	Genotype	Predicted effect on protein	Age (gender)	Age of first symptoms	Phenotype at onset	Pattern of muscle weakness; other clinical features	Cardiac involvement?	Contractures?	Independent locomotion? (age)	Loss of ambulation? (age)	CK levels (U/l)	Muscle biopsy	Laminin-α2 IHC [1]	Brain changes (MRI)
F.I-P1	c.2461A > C + c.7750-1713_7899-2154del	p.Thr821Pro + p.Ala2584Hisfs*8	42 yrs (F)	Third decade	Migraine-like headaches for 8 mo. Complaints of limb weakness and walking difficulties	Mild generalized muscular atrophy and tetraparesis (4+/5 grade), feet dorsiflexion (4/5). Paresis of trunk and neck flexion, cannot do sit-ups or lift head while in supine position.	N	N	Y	N	n.p.	Moderate myopathic changes, with discrete endomysial fibrosis	Normal	WMC
F.I-P2	c.2461A > C + c.7750-1713_7899-2154del	p.Thr821Pro + p.Ala2584Hisfs*8	50 yrs (M)	Forth decade	Running difficulties and progressive lower limb weakness	Proximal paresis (4/5 grade) in upper limbs, distal and proximal paresis in lower limbs. Paresis of trunk and neck flexion (grade 2 and 4+, respectively). Lordosis and myopathic gait with slight steppage.	N	N	Y	N	n.p.	Myopathic changes with fiber necrosis, also "ragged red fibers" and large number of COX negative fibers.	Normal	WMC
F.II-P3	c.2461A > C + c.3976C > T	p.Thr821Pro + p.Arg1326*	15 yrs (F)	18 mo	Running difficulties and stairs	Proximal paresis (2/5 grade) in upper limbs and in lower limbs (3/5 grade), neck flexion (grade 2/5).	N	Y	Y (15 mo)	N	839	Dystrophic changes	Normal	WMC
F.II-P4	c.2461A > C + c.3976C > T	p.Thr821Pro + p.Arg1326*	11 yrs (F)	5 yrs	Facial fatigue	Proximal paresis (2/5 grade) in upper limbs and in lower limbs (4/5 grade), neck flexion (grade 1/5).	N	N	Y (14 mo)	N	2466	n.p.	n.p.	n.p.
F.III-P5	c.2461A > C + c.1854_1861dup	p.Thr821Pro + p.Leu621Hisfs*7	33 yrs (F)	Childhood	Running difficulties. Difficulty in getting out of a bed	Proximal paresis (4-3/5 grade) in lower limbs. Paresis of trunk and neck flexion (grade 3/5). Lordosis and myopathic gait.	N	N	Y	N	~2,000	Dystrophic changes	Partial deficiency	n.p.
F.IV-P6	c.2461A > C (hom.)	p.Thr821Pro	71 yrs (M)	Childhood	Gait impairment	LGMD initially suspected. Proximal tetraparesis (grade 4/5, 4-/5 in lower limbs). Moderate intellectual disability (last 2 yrs).	Y [2]	N	Y	Y (last 2 years)	579	Moderate dystrophic changes associated to angulated atrophic fibers and nuclear clumps	Normal	WMC, thalamus and pons involvement

Notes. F: female; hom.: homozygous; LGMD: limb-girdle muscular dystrophy; M: male; MD: muscular dystrophy; mo: months; N: no; n.p.: not performed; U: unknown; WMC: white matter changes; Y: yes; yrs: years. [1]: Clone: Mer3/22B2 (Leica Biosystems, Newcastle upon Tyne, United Kingdom); [2]: Left ventricle hypokinesia of unknown cause, normal ejection fraction.

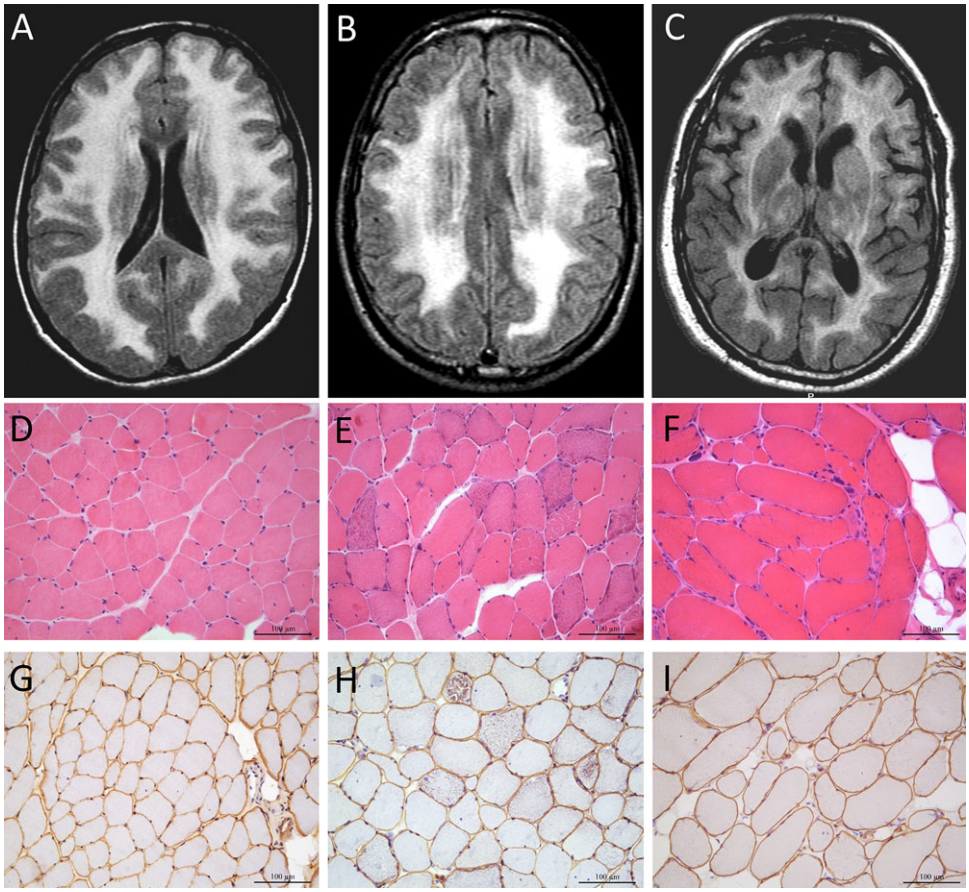

FIGURE 3 Brain magnetic resonance imaging (MRI) and muscle neuropathology results. Patient P1: (a) brain MRI (FLAIR) shows typical white matter changes (WMC) with normal structural cerebral cortex changes; (d) moderate myopathic changes with discrete endomysial fibrosis in hematoxylin eosin (h & e) staining, and (g) normal immunohistochemistry (IHC) for laminin-α2. Patient P2: (b) brain MRI (FLAIR) with WMC and normal cerebral cortex; (e) myopathic changes with necrotic fibers and several "ragged red fibers" (h & e), and (h) normal IHC for laminin-α2. Patient P6: (c) WMC in brain MRI (FLAIR); (f) dystrophic changes (necrotic fibers under myophagocytosis, fiber splitting and hypersegmentation, and fat substitution) and mild neurogenic features (atrophic angulated fibers and nuclear clumps), and (i) normal IHC for laminin-α2

patient cohort. This missense substitution was initially identified in two patients with atypical presentations (Marques et al., 2014), prior to the six patients described above. A further three patients, from two unrelated families with Portuguese ancestry, have also been reported by other groups: one patient from Canada (with #102482 in LAMA2-LOVD), and two brothers studied in France (Nelson et al., 2015). Thus far, all patients are reported to have milder muscle weakness and the majority were initially classified as possible LGMD or EDMD. To evaluate if this missense variant could account for additional uncharacterized cases, we screened an irreversibly anonymized group of 239 myopathic Portuguese patients with clinical presentation is compatible with LGMD or EDMD. Variant screening was performed by restriction fragment length analysis (RFLA, Supporting Information IV), since the c.2461A > C change creates a new restriction site for *Hpy*CH4III (Supporting Information Figure S4). Positive samples were confirmed by Sanger sequencing. A total of seven patients carried this missense substitution (2.9% of the cohort), three of which were homozygotes and four were heterozygotes (2% of all disease alleles). To further ascertain the genotype of the four patients carrying the c.2461A > C variant in heterozygosity, the entire coding sequence of the *LAMA2* gene was sequenced. In all patients an additional heterozygous variant was detected. Three were previously identified in other (MDC1A) patients: c.4739dup (p.Leu1581Profs*5; Oliveira et al., 2008), c.3372dup (p.Cys1125Metfs*4; patient #103970 in Table 1) and a missense variant c.32T > C (p.Leu11Pro) listed in ClinVar (RCV000157587.1) as being disease associated. The fourth variant, c.6707G > A, is also new and was interpreted as a VUS; it predictably gives rise to a missense change (p.Arg2236Lys) and/or may have an effect on splicing (r.spl?; Table 2, patient #103971).

Since the c.2461A > C variant was not listed in variant population databases, its prevalence was estimated in control individuals using the aforementioned RFLA-screening strategy. For this study, we randomly selected and irreversibly anonymized 1,100 out of a total of 11,000 samples previously analyzed in the laboratory. These were residual samples from genetic studies for diseases unrelated with neuromuscular disorders that are performed on a nationwide basis. The c.2461A > C variant was identified in one of these samples, in heterozygosity. Its allelic frequency in the general population was estimated as 0.0452% (1/2,200), which explains the relatively high prevalence of this variant among Portuguese patients with *LAMA2*-MD.

Overall, the presented data reinforces that it is diagnostically important to consider *LAMA2* gene involvement not only in CMD

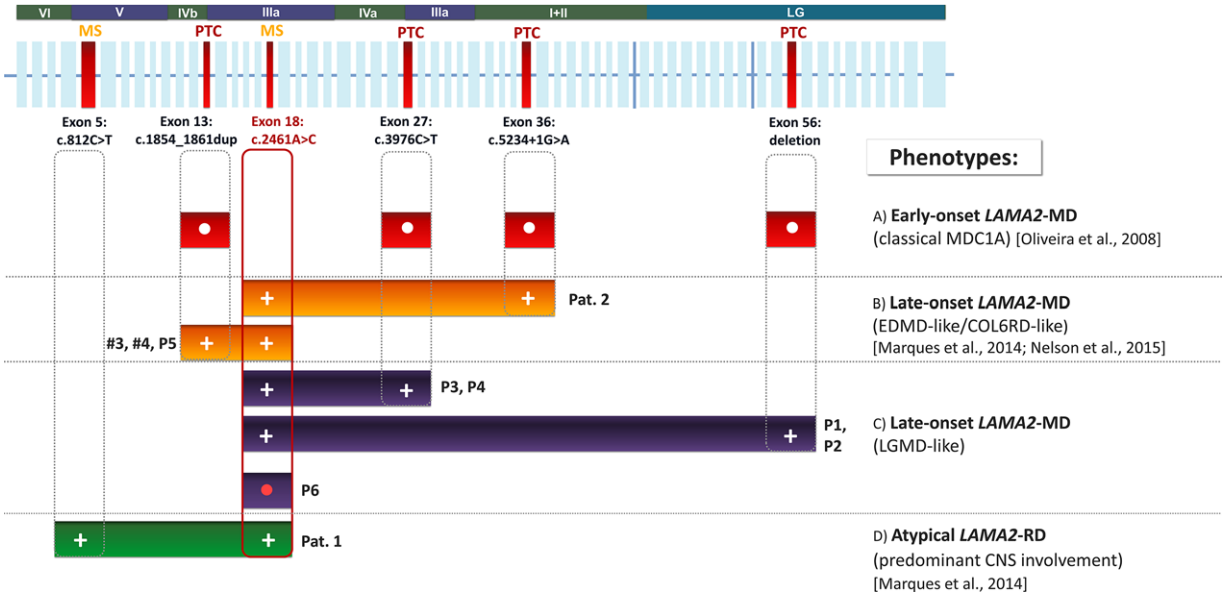

FIGURE 4 Different phenotypes in laminin-α2-related muscular dystrophy (*LAMA2*-MD) found in association with the c.2461A > C (p.Trp821Pro) variant. (a) Early-onset (classical muscular dystrophy type 1A [MDC1A]): no independent ambulation; muscle biopsy shows dystrophic features and no labeling for laminin-α2 in immunohistochemistry (IHC). Several patients reported in Oliveira et al. (2008). (b) Late-onset (Emery–Dreifuss muscular dystrophy [EDMD]/COL6-RD-like): rigid spine syndrome; cardiac involvement in some patients; walking difficulties; dystrophic features in MD, normal and irregular laminin-α2 staining in IHC. Pat.2—patient 2 (Marques et al., 2014); cases #3, #4 (Nelson et al., 2015); P5 (this work). (c) Late-onset (limb-girdle muscular dystrophy (LGMD)-like): slow progression; dystrophic features, normal and irregular laminin-α2 staining in IHC, walking difficulties later in life; P1–4, P6 (this work). (d) Atypical *LAMA2*-RD: predominant central nervous system (CNS) involvement, (occipital agyria, white matter changes (WMC), epilepsy); increased variability of muscle fiber diameter and irregular laminin-α2 staining in IHC. Pat.1—patient 1 in Marques et al. (2014). Genotype–phenotype correlations suggest that the classical MDC1A presentation is explainable by variants causing premature termination codons (PTC) in both disease alleles. While late-onset *LAMA2*-MD are more likely to be associated with missense (MS) substitutions
Note. •: homozygous; +: Heterozygous.

patients, but also as a possible cause of MD with onset beyond childhood and even in adulthood. The association between *LAMA2* and this later onset phenotype was not fully established, considering the limited number of cases reported so far. Nonetheless, it is advisable to include *LAMA2* in the list of candidate genes for MDs (LGMD or EDMD). The p.Trp821Pro missense variant constitutes an interesting genotype–phenotype linker, as it may give rise to different phenotypes depending on the variant found in the second allele (Figure 4).

6 | DIAGNOSTIC RELEVANCE

Molecular defects in the *LAMA2* gene are the main genetic causes (~30%) of CMDs in most countries, except for Japan where Fukuyama-type CMD has the highest prevalence, due to a frequent founder mutation in the *FKTN* gene (Kobayashi et al., 1998). Besides the clinical examination, the clinical diagnostic workup of CMDs conventionally relies upon performing a muscle biopsy (Bönnemann et al., 2014). In addition to standard staining methods, muscle pathology analysis includes a panel of antibodies for IHC against proteins involved in MD (laminin-α2, sarcoglycans, dystrophin, and dysferlin). Three different commercial antibodies are currently available for laminin-α2 IHC studies: clone 5H2 detects the 80 kDa protein (C-terminal region), clone Mer3/22B2 detects the 300 kDa product (N-terminal region), and clone 4H8-2 clone, which also recognizes the N-terminal domain. The diagnostic sensitivity of IHC is extremely high for typical MDC1A cases, where complete deficiency would be detectable regardless of the antibody used for analysis. The milder *LAMA2*-MD cases are more challenging as often only a partial deficiency is often documented. Moreover, depending on the underlying molecular defects, this IHC deficiency may not be consistent for the different antibodies (Cohn, Herrmann, Sorokin, Wewer, & Voit, 1998). N-terminal antibodies usually have higher sensitivity for cases with partial laminin-α2 deficiency, as there was a relatively intact labeling with the antibody for the 80 kDa fragment, when compared with that using the other antibodies (Cohn et al., 1998). It is therefore advisable to include at least two different antibodies against laminin-α2 in order to increase IHC sensitivity. In a small fraction of CMD patients there is also irregular labeling or partial laminin-α2 deficiency. There is some degree of genetic heterogeneity among these patients, depending on whether it is a primary or a secondary deficiency. To distinguish between these two possibilities, antibodies against glycosylated residues of α-DG and laminin-α4/5 may be effective. If changes are detected in α-DG, this would indicate a defective glycosylation pathway and involvement of other loci. On the other hand, normal α-DG labeling and overexpression of laminin-α4/5 (a compensatory gene expression mechanism) is suggestive of a primary laminin-α2 deficiency.

Brain MRI performed beyond the first 6 to 12 months is also an important diagnostic resource for CMDs. As previously mentioned all *LAMA2*-related MD patients have brain WMC, consisting in bilateral hyperintensity signal on T2-weighted and FLAIR MRI, in periventricular areas and subcortical cerebral hemisphere (Quijano-Roy et al., 2012). These findings alone should be an indication to perform *LAMA2* genetic testing. As demonstrated by this work and previously suggested by Gavassini et al. (2011), it is diagnostically relevant to perform brain MRI in uncharacterized LGMD patients. This could be performed even during adulthood, as these typical brain changes will persist throughout life. Brain MRI is especially relevant for "atypical" or mild MD cases where IHC for laminin-α2 has a lower diagnostic yield.

Considering the size and number of exons in the *LAMA2* gene, its genetic analysis has been simplified, more than a decade ago, with the introduction of automatized sequencers (fragment analyzers) for Sanger sequencing and the use of universal-tailed primers. Gene sequencing is undoubtedly the approach with the highest sensitivity for *LAMA2* analysis, detecting approximately 80% of disease-associated variants. Based on the variant data collected, there is a significant frequency (~18%) of large deletions and duplications. The genetic study should therefore be complemented with other molecular techniques such as MLPA or array-CGH.

Data available in the LSDB and population-specific cohorts can help to optimize the *LAMA2* genetic analysis. This was exemplified in a Portuguese CMD patient cohort where a 3-tier genetic test was proposed (Oliveira et al., 2014): (a) sequencing a small set of selected exons where the majority of point mutations are located (based on a specific population or ethnical group variant data); (b) sequencing the remaining *LAMA2* exons; and (c) MLPA analysis or array-CGH.

The introduction of next-generation sequencing technology (NGS, or massive parallel sequencing) has remodeled genetic analysis strategies, especially in genetically heterogeneous conditions such as the MDs. Distinct NGS applications such as gene panels or whole-exome sequencing (WES) can be extremely useful to address diagnostically difficult cases. In fact, novel cases with milder *LAMA2*-related phenotypes recently reported in the literature have been solved resorting to NGS (Dean, Rashid, Kupsky, Moore, & Jiang, 2017; Ding et al., 2016; Harris et al, 2017; Kim et al., 2017).

The impact of NGS technology is also reflected in five patients described in this work: (ID#s: 102662, 132012, 132013, 132015, 132025 in table I), whose disease-associated variants listed were identified by NGS gene panels. One further patient (ID# 103207 in Table 1) demonstrates the utility of NGS to address genetic and clinical heterogeneity. This is a patient with an LGMD phenotype and ID, who has remained without genetic characterization for several years. The patient, currently 14 years of age, had delayed motor development (started walking at 31 months of age), lumbar lordosis, and elevated CK levels (~1300 U/l). Muscle biopsy performed at 6 years of age (in another clinical center where she was initially followed) revealed dystrophic features and normal IHC results for dystrophin and sarcoglycans. Genetic analysis of *FKRP*, *CAPN3*, *LMNA*, and *DMPK* genes were negative. The patient was studied by WES as previously reported in a similar research (Oliveira, Martins, Pinto Leite, Sousa, & Santos, 2017). As a first approach, WES data analysis was restricted to a set of genes known to be associated with muscle diseases (Supporting Information IV). Within the list of filtered-in variants, two heterozygous variants were identified in *LAMA2* (Supporting Information Figure S5). The first was the c.1854_1861dup variant, previously reported as disease associated in several MDC1A patients, and the second was a novel splicing variant c.819+2T > C located in the donor splice-site of intron 5 (Table 1). To further characterize the effect of this splice-site variant, a muscle fragment available from patient ID# 102735 (shares the same variant) was used (Supporting Information II). *LAMA2* transcript analysis by RT-PCR showed the presence of multiple aberrant products that, upon sequencing, were attributed to multiple skipping events involving exons 5 to 7 (Table 1, Supporting Information Figures S1 and S2). Study of the patient's parents confirmed compound heterozygosity, as each progenitor carried a different *LAMA2* variant. Brain MRI performed after WES analysis, revealed WMC but not configuring the typical pattern found in MDC1A cases. In this patient, axial T2 and FLAIR revealed small focal white matter hyperintensities in the subcortical part of brain, more specifically in the frontal, temporal-anterior, parahyppocampus, and insula regions with sparing of the internal capsule and corpus callosum (data not shown).

Finally, as demonstrated by five cases listed in Table 1 (ID#s 102324, 102369, 102378, 132014, and 132015), a small percentage of patients were found to have only one heterozygous *LAMA2* disease-causing variant. This could be attributed to deeply placed intronic variants affecting splicing or variants located in the gene's promotor region, both of which are not covered by conventional sequencing, gene panels, or even WES. Here, a more comprehensive NGS approach such as WES and/or RNA sequencing may ultimately provide a final answer to such cases with incomplete genotyping.

7 | FUTURE PROSPECTS

Although there is an increasing recognition of the involvement of *LAMA2* disease-associated variants in the genetic etiology of muscular dystrophies, the incidence is probably still underestimated. To improve the diagnosis of these cases, it is necessary to include both brain MRI and to evaluate the expression of laminin-α2 in muscle by IHC. These two approaches are often not considered in the clinical workup of patients with (non-congenital) myopathies. NGS can also contribute toward the identification of further cases. However, the interpretation of variants from such studies often leads to their classification as VUS, which considerably limits the clinical utility of these genetic data.

As for further research, it is necessary not only to continue to document clinical data and *LAMA2* variants to obtain further genotype–phenotype correlations, but also to develop strategies for functional analysis and validation of new variants, especially those predictably of the missense type. This task may be complex, as variants might affect several key aspects of the laminin-211 life-cycle: (a) posttranslational modification, (b) protein translocation and secretion process, (c) interaction with membrane-specific receptors, and (d) variety of molecular partners in the BM, possibly some yet to be identified. One strategy for functional analysis would imply obtaining a biological sample from the patient by an invasive procedure (e.g., muscle or

skin biopsy), expanding cells through *in vitro* culture, and performing protein–protein interaction studies, such as pull-down assays using a battery of different bait-proteins known to interact with laminin-α2. Failure to detect a particular interaction would indicate a deleterious effect. To enable such studies, further research should primarily focus in a comprehensive search for domain-specific interactions, which could be accomplished by high-throughput proteomics analyses. An assay for those variants specifically affecting domains involved in laminin polymerization has been reported (Cheng, Champliaud, Burgeson, Marinkovich, & Yurchenco, 1997; Hussain, Carafoli, & Hohenester, 2011). Basically, a mixture of wild-type with the mutated form of laminin would show a failure in establishing normal polymerization levels. Here, the limiting step would be generating and purifying sufficient amounts of proteins to conduct these *in vitro* studies.

As laminin-α2 is not confined to muscle or brain cells, in a transgenic mouse model with deficient laminin-α2 it was shown that the loss of this protein caused disruption of the apical ectoplasmic specialization-blood–testis barrier, and leading to male infertility (Häger, Gawlik, Nyström, Sasaki, & Durbeej, 2005). The laminin-α2 in testis was further implicated in the regulation of an axis that functionally links the BM to the blood–testis barrier of Sertoli cells (Gao et al., 2017). Considering that human infertility has not been linked to laminin-α2, it would be relevant to evaluate male reproductive issues in late-onset *LAMA2*-MD patients.

One of the most important aspects concerning *LAMA2*-MD is the development of a suitable treatment for this condition. Several approaches have been proposed, developed, and tested in laminin-α2-deficient mice and zebrafish models (reviewed by Durbeej, 2015; Wood & Currie, 2014). One particularly effective approach targets extracellular matrix modulation as a way to ameliorate MDC1A. Here, strategies aim to improve muscle viability, through the augmentation of residual functionality within the cellular system, such as upregulation of other laminins (α4 or α1) and integrin-α7 (Wood & Currie, 2014). However, with laminin-411 there are some limitations for BM repair, since this laminin only forms a trimeric structure, lacking capacity to further self-polymerize into superstructures such as those derived from laminins-211 or -111. Overall there are some hurdles toward its applicability, namely the large size of laminins, which make its delivery to target locations extremely challenging. An effective way to address this problem is to use shorter engineered proteins, such as the chimeric laminin/nidogen protein or mini-agrin, shown to be effective in a *LAMA2*-MD mouse model (dy^W/dy^W; McKee et al., 2017; Reinhard et al., 2017). Probably in a near future, we will witness a new generation of laminin-binding proteins that, depending on the underlying genetic defects, are able to replace defective domains of laminin and promote the assembly of a stable and fully functional BM.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

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